Original Article

Optimizing Culture Condition for High Frequency Regeneration *in Vitro* Tissue Culture in *Daucus Carota* L. and Genetic Stability Assessment

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ABSTRACT

Carrot is well known for medicinal purposes. For this reason, optimization of tissue culture is necessary and the first step for this purpose. In the present study, an efficient *in vitro* direct and indirect regeneration of *Daucus carota* L. was performed. The root, shoot, leaf and nodal explants were cultured on Murashige and Skoog (MS) medium supplemented with different concentration and combinations of plant growth regulators (PGRs). Direct multiple shoot regeneration was induced on seventeen media culture varied in the type of culture medium and PGRs; the MS medium containing 1 mg/l Benzylaminopurine (BAP) + 2 mg/l NAA (Naphthylacetic Acid) was the best medium for direct shoot regeneration. In indirect regeneration the, highest callus induction, embryogenesis and the shooting rate obtained from shoot segments followed by leaf segments cultured on the MS medium containing 2 mg/l NAA, respectively. The highest rooting percentage (95%) was recorded on the MS and ½MS medium both containing 1 mg/l NAA. Medium 1 including MS + 2 mg/l NAA + 1 mg/l BAP was the best medium for direct regeneration, and medium 6 including MS + 2 mg/l NAA, was the best medium for indirect regeneration. Percentage of adaptation was 45%. Intra shoot variability of the shoot forming capacity indicated in the Nantes depended on the shoot position. Shoot differentiation from leaf tissue indicated that highest shoot- forming capacity was obtained from two nearest explants to apex of 1 mg/l NAA of 2 mg/l. The results of genetic fidelity with ISSR markers indicated that of 6 regeneration plants, one plant was not same as mother plants in genetic stability, but five regeneration plants were the same as mother plant in genetic stability. This result showed genetic stability in mother plant and regeneration plants was very high. In this research, the highest amount of callus induction, embryogenesis and shooting was obtained.

1. Introduction

Carrot (*Daucus carota* L.) is a member of family Apiaceae and is well known for medicinal purposes [1, 2]. This plant is a rich source of the fat- soluble hydrocarbon, β carotene which is the precursor of Vitamin A [3]. The phytochemicals in carrots play an important nutritional role in human health [4].

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Tissue culture is important for the conservation of genetic resources, micropropagation, cloning and genetic manipulation studies for molecular farming [5]. Improving or modifying the trait or creating a new trait in plants is done by genetic engineering. Also, carrots are used as a plant model in order to produce vaccines by molecular farming [5]. Jaime *et al*. used carrot for producing a low-cost vaccine candidate against synucleinopathies [6]. In another research, carrot was applied for producing edible plant-based vaccines [7, 8]. Marquet-Blouin *et al.,* used carrot for vaccine production against virus hemagglutinin [5] and Monreal-Escalante *et al.,* worked on it for the production of vaccine against porcine cysticercosis [9]. Therefore, optimization of tissue culture of carrot is necessary and important. Until now for optimization of tissue culture of carrot, different types and concentrations of plant growth regulatory and different of explants have been applied. Rabiei *et al*., showed different plant growth regulatory of 2,4-D essential for callus induction initiation and growth of callus. Another research indicated more effect for regeneration [10]. Edwin *et al.,* showed the use of low concentration of cytokinin was essential for embryogenesis (59%) [11]. Cristina Tavares *et al.,* showed 2,4-D for somatic embryogenesis [12]. Percentage of embryogenesis in this study was 90%. In another research, NAA was used for callus induction [13]. Cristina Tavares *et al.,* used BA for proliferation [12]. NAA was used for rooting [13]. Kiszczak *et al.* used B5 medium containing 2,4-D and NAA at 0.1 $g \cdot L^{-1}$ [14]. Shmykova *et al.,* used NLN-13 medium supplemented with 0.2 mg/L 2,4D and 0.2 mg/L kinetin for embryogenesis and 0.1 mg/L of kinetin for regeneration [15]. In another research, anther was used for regeneration [16] and root [17]. Production of high similar plants by old methods is costly and time-consuming. Nowadays, tissue culture is used to produce a large number of similar plants. Genetic diversity may be created in plants produced by tissue culture. But genetic stability is very important in producing plants and the aim is the production of plants that are similar in genetic stability [18]. Raji *et al.,* used ISSR and RAPD markers for indicating genetic fidelity in plant mother and regeneration plant [19]. A study compared between Pseudoperonospora cubensis isolates from Europe and Asia by using ISSR and SRAP molecular markers for indicating polymorphic [18]. Genetic uniformity of transgenic cottons (Bt and chitinase lines), using RAPD, ISSR molecular markers, the study has been scrutinized [19]. For this purpose, to develop a more efficient protocol, we tested different explants, different plant hormone combinations and different concentration for their ability to callus induction, embryogenesis and regeneration. Additionally, regeneration plants can be produced from plant mother which may have somaclonal variation. Somaclonal preservation of the genetic diversity of the regeneration plants and mother plant is important. For this reason, ISSR and RAPD primers were used [19]. The results of this study can be used in genetic transformation efficiency, in vitro tissue culture and production of secondary metabolite.

As mentioned before, carrot is used as a plant model for molecular farming and vaccine production. For this aim, the first step is optimization of tissue culture of carrot. The purpose of this research was to obtain the highest percentage of callus induction, embryogenesis and shooting. The next goal was the assignment of genetic fidelity between mother plant and regeneration plant of mother plants.

Materials and Methods

Preparation of Explants

Seeds of carrot (*Daucus carota* L. cv. Nantaise) were prepared from Seed and Plant Improvement Institute (Karaj, Iran). For seed culture, the healthy seeds were sterilized by immersing them in the solution of 70% ethanol for 1 min and then immersing in the solution of sodium hypochlorite (5%) for 20 min and finally by rinsing three times with sterile water [12]. The seeds were cultured on ½MS medium supplemented with 3% sucrose and solidified with 0.8% agar. pH of the medium was adjusted to 5.7 before autoclaving. Cultures were maintained at 25 ± 2 Ċ exposed to 16 hours light and 8 hours dark per day [12].

The seeds were germinated after two to three weeks of culture with normal seedling. Explants including root, stem, leaf and nodal from the *in vitro* grown seedlings were excised in 3 mm pieces and cultured on MS medium supplemented with different concentrations of plant growth regulators including NAA, BAP, Kin and 2,4- D alone or in combination together (Table 1) [17]. Callus induction, embryogenesis, regeneration and rooting response were examined under the given cultured condition (Tables 1, 2, 3 and 4). Cultures were sub-cultured into fresh media twice every four weeks [13]. The regenerated plantlets were transferred to pots including perlite in order to grow into normal plants.

Table 1. The used mediums for callus induction

Medium	Composition of medium
1	MS + 30 g/l sucrose + 2 mg/l NAA + 1 mg/l BAP
2	MS + 30 g/l sucrose + 1 mg/l NAA
3	MS + 30 g/l sucrose + 0.2 mg/l 2,4-D
4	MS + 30 g/l sucrose + 1 mg/l 2,4-D + 0.1 mg/l kin
5	MS + 30 g/l sucrose + 0.5 mg/l BAP
6	MS + 30 g/l sucrose + 2 mg/l NAA
7	MS + 30 g/l sucrose + 1 mg/l 2,4-D
8	MS + 30 g/l sucrose + 1 mg/l 2,4-D + 21.5 µg/l kin
9	MS + 30 g/l sucrose + 0.5 mg/l 2,4-D
10	MS + 30 g/l sucrose + 1 mg/l 2,4-D + 21.5 µg/l kin (in dark)
11	MS + 30 g/l sucrose + 1 mg/l 2,4-D
12	MS + 30 g/l sucrose + 0.3 mg/l BAP + 2 mg/l NAA
13	MS + 30 g/l sucrose + 0.2 mg/l 2,4-D
14	MS + 30 g/l sucrose + 1 mg/l 2,4- D
15	MS + 30 g/l sucrose + 0.2 mg/l 2,4-D + 0.2 mg/l BAP
16	MS + 30 g/l sucrose + 0.2 mg/l 2,4-D + 0.2 mg/l Kin
17	MS + 30 g/l sucrose + 0.5 mg/l 2,4-D + 0.5 mg/l BAP
18	MS + 30 g/l sucrose + 1 mg/l 2,4-D + 1 mg/l Kin
19	MS + 30 g/l sucrose

Table 2. The used mediums for embryogenesis

Medium	Composition of medium
1	MS + 30 g/l sucrose + 2 mg/l NAA + 1 mg/l BAP
2	MS + 30 g/l sucrose + 1 mg/l NAA
3	MS + 30 g/l sucrose + 0.2 mg/l 2,4-D
4	MS + 30 g/l sucrose + 1 mg/l 2,4-D + 0.1 mg/l kin
5	MS + 30 g/l sucrose + 0.5 mg/l BAP
6	MS + 30 g/l sucrose + 2 mg/l NAA
7	MS + 30 g/l sucrose + 0.5 mg/l 2,4-D
8	MS + 30 g/l sucrose + 1 mg/l 2,4-D + 21.5 µg/l kin
9	MS + 30 g/l sucrose + 0.5 mg/l 2,4-D
10	MS + 30 g/l sucrose + 1 mg/l 2,4-D + 21.5 µg/l kin (in dark)
11	MS + 30 g/l sucrose
12	MS + 30 g/l sucrose + 0.3 mg/l BAP + 2 mg/l NAA
13	MS + 30 g/l sucrose + 0.2 mg/l 2,4-D
14	MS + 30 g/l sucrose + 1 mg/l 2,4- D
15	MS + 30 g/l sucrose + 0.2 mg/l 2,4-D + 0.2 mg/l BAP
16	MS + 30 g/l sucrose + 0.2 mg/l 2,4-D + 0.2 mg/l Kin
17	MS + 30 g/l sucrose + 0.5 mg/l 2,4-D + 0.5 mg/l BAP
18	MS + 30 g/l sucrose + 1 mg/l 2,4-D + 1 mg/l Kin

19	MS + 30 g/l sucrose

Table 3. The used mediums for regeneration

Medium	Composition of medium
1	MS + 30 g/l sucrose + 2 mg/l NAA + 1 mg/l BAP
2	MS + 30 g/l sucrose + 1 mg/l NAA
3	MS + 30 g/l sucrose + 0.2 mg/l 2,4-D
4	MS + 30 g/l sucrose + 1 mg/l 2,4-D + 0.1 mg/l kin
5	MS + 30 g/l sucrose + 0.5 mg/l BAP
6	MS + 30 g/l sucrose + 2 mg/l NAA
7	MS + 30 g/l sucrose
8	MS + 30 g/l sucrose + 1 mg/l 2,4-D + 21.5 µg/l kin
9	MS + 30 g/l sucrose + 0.5 mg/l 2,4-D
10	MS + 30 g/l sucrose + 1 mg/l 2,4-D + 21.5 µg/l kin
11	MS + 30 g/l sucrose
12	MS + 30 g/l sucrose + 0.3 mg/l BAP + 2 mg/l NAA
13	MS + 30 g/l sucrose + 0.2 mg/l 2,4-D
14	MS + 30 g/l sucrose + 1 mg/l 2,4- D
15	MS + 30 g/l sucrose + 0.2 mg/l 2,4-D + 0.2 mg/l BAP
16	MS + 30 g/l sucrose + 0.2 mg/l 2,4-D + 0.2 mg/l Kin
17	MS + 30 g/l sucrose + 0.5 mg/l 2,4-D + 0.5 mg/l BAP
18	MS + 30 g/l sucrose + 1 mg/l 2,4-D + 1 mg/l Kin
19	MS + 30 g/l sucrose

Table 4. The used mediums for rooting

Medium	Composition of medium
1	MS + 30 g/l sucrose
2	MS + 30 g/l sucrose + 1 mg/l NAA
3	MS + 30 g/l sucrose + 0.5 mg/l NAA
4	½MS + 30 g/l sucrose
5	½MS + 30 g/l sucrose + 1 mg/l NAA
6	½MS + 30 g/l sucrose + 0.5 mg/l NAA

Intra- shoot variability of the shoot-forming capacity

The shoot- forming capacity difference was determined from six shoot positions. In this research 2 mg/l NAA was used alone and 2 mg/l NAA and 1 mg/l BAP together.

Shoot differentiation from leaf tissue

To indicate the position of leaf efficient on shoot-forming capacity 1 mg/l NAA was used.

Assessment of genetic stability

To determine the genetic stability of regenerated plant compared with plant mother, 18 primers of ISSR for amplification of the DNA analysis was applied (Table 5) [22].

Table 5. ISSR primer used this experiment

Primer	Sequence primer
ISSR 1	TTCTTCTTCTTCTTCTTCG
ISSR 3	TCTCTCTCTCTCTCTCC
ISSR 5	TCTCTCTCTCTCTCTCT
ISSR 7	GAAGAAGAAGAAGAAGAAG

ISSR 8	GGAAGAAGAAGAAGAAGAA
ISSR 9	GAAGAAGAAGAAGAAGAAC
ISSR 10	CACACACACACACACAG
ISSR 12	CACACACACACACACAA
ISSR 13	CACACACACACACACAT
ISSR 14	CTCTCTCTCTCTCTCT
ISSR 15	CTCTCTCTCTCTCTCTCA

In this experiment, one mother plant and 6 regeneration plants of this mother plant were utilized. The cycle program followed these steps: 35 cycles at 94 Ċ for 1 min (denaturation), then at 53, 55, 59 Ċ for 1 min (annealing) and then 72 Ċ for 1 min (extension) and the end final extension step at at 72 Ċ for 5 min. This test was repeated three times.

Statistical Analysis

In order to see whether the seedling micropropagation depends on the type and concentration of plant growth regulatory and the type of explants, ANOVA was computed. Four stages of micropropagation including callus induction, embryogenesis, regeneration and rooting, were based on Completely Randomized Design (CRD) with 3 replications. Data were analyzed using SPSS software. Significantly different means were identified using Tukey's test (P = 0.05).

Results

Seed germination and Callus induction

The seed germinated after three days in ½ MS medium (Figure 1A). The seedling grew sufficiently after three weeks. Calli were formed three weeks after tissue culture of explants (Figure 1B). The results related to Calli Fig. 2 showed that Callugenesis of root, leaf and shoot explants was affected by the used treatments and was significant at both 1% and 5% levels, while Callugenesis of nodal explants was not affected by the used medium.

In the study using media, eight media, including 3, 4, 6, 7, 12, 13 and 17 create calli of root explants 3 and 4 media of shoot explants, media 4 and 16 of nodal explants and media 7, 12, 16 and 17 from leaf explants turned brown and died (Figure 1C).

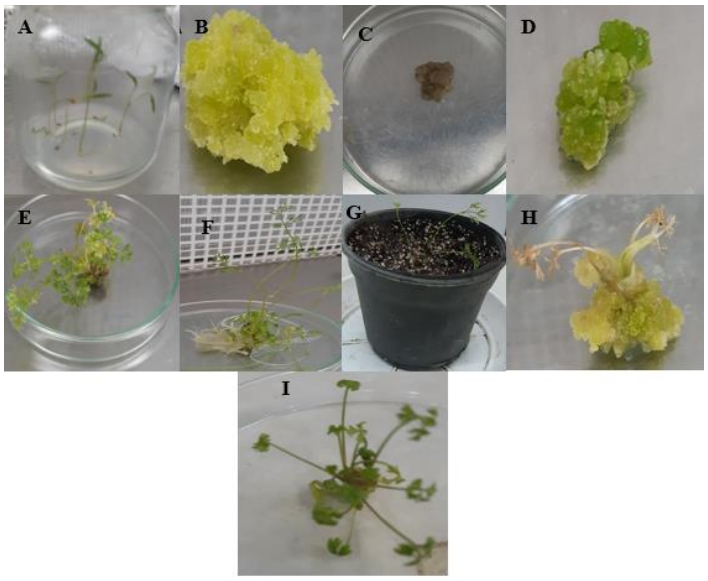

Figure 1. Callus induction, embryogenesis, regeneration and rooting potential derived from different explants. A. The Seed germination after 3 weeks. B. Callugenesis created after 3 weeks. C. Brown and died callus of leaf explants. D. Embryogenesis created after 5 weeks. E. Shooting created after 8 weeks. F Rooting after ten weeks. G. Regenerated plants transferred to pots. H. plant

obtained from nodal explants that died in direct regeneration. I. Plants obtained from nodal explants in direct regeneration.

The highest percentage of callus induction and calli of suitable size and growth were produced in media including medium 1 of shoot explant, medium 2 of root and leaf explants, medium 6 of shoot and leaf explants and medium 9 of shoot explant (Table 1) (Figure 2).

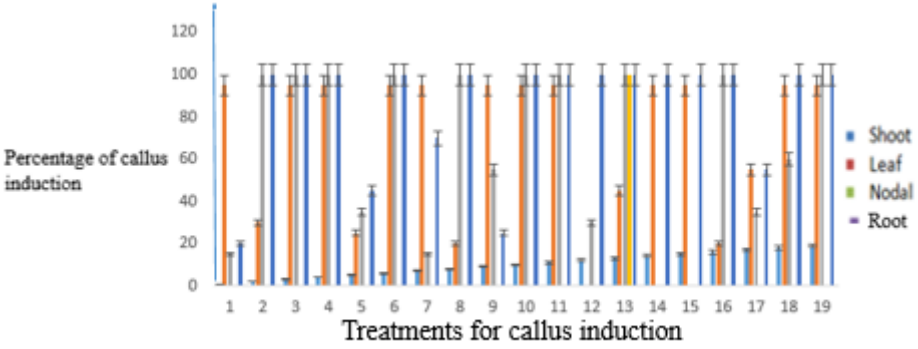

Figure 2. Percentage of callus induction after 3 weeks.

Embryogenesis

Embryogenesis was formed five weeks after tissue culture of explants (Figure 1D). The results of analysis indicated (Figure 3) that the used treatment for embryogenesis of explants root, leaf and shoot at 1% level was significant, but using nodal explants, embryogenesis formation was not significant by the used treatment.

The highest percentage (95%) of embryogenesis and embryos of suitable size and growth were produced in media including 14, 15, 18 and 19 from shoot explants and media 6 and 19 from of leaf explants (Table 2) (Figure 3), whereas the lowest percentage (0%) of embryogenesis was related to media including 3, 11, 12 and 16 (Table 2). In all media no embryos of nodal explants were formed.

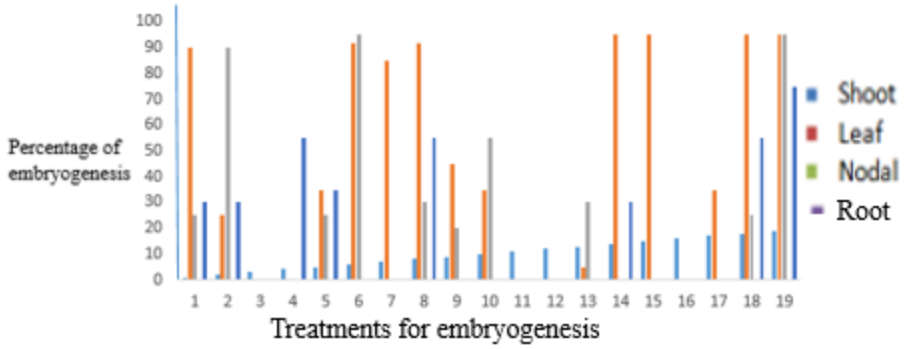

Figure 3. Percentage of embryogenesis after 5 weeks in different treatments

Shooting

Shooting was formed three weeks after embryogenesis (Figure 1). The used explants (root, shoot, leaf and nodal) for shooting showed significant at 1% level by the used treatment. In this study, in medium 6, the percentage of the shooting of shoot explants was 90% (Table 3) (Figure 4. Figure 1E).

In this study, the percentage of multiple shoot by direct nodal explants in 1 and 6 media was 95% (Table 1) (Figure 1I).

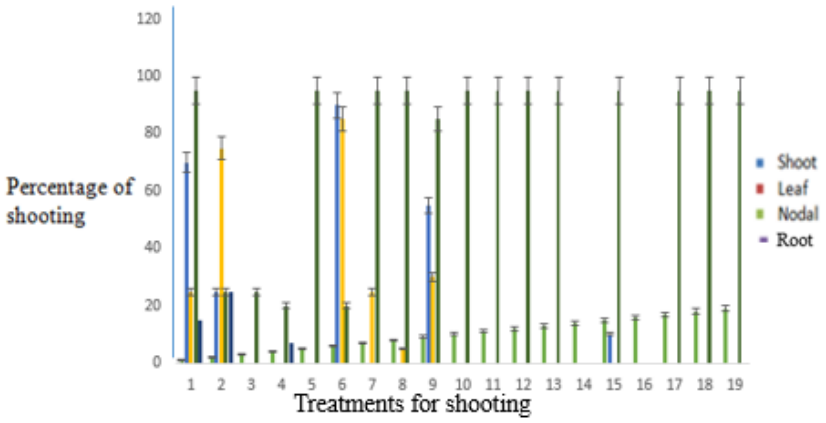

Figure 4. Percentage of shooting after 8 weeks.

All plants obtained from nodal explants in media 3, 11 and 13 were not in good condition and 95% of plants obtained from nodal explants in media 17 and 19 died (Figure 4I). But plants obtained of nodal explants in other media had normal condition (Figure 1I).

Rooting

Seedlings were rooted three to five weeks after being placed in rooting medium (Fig. 1, F). Of six medium used for rooting, in 1 (MS + 30 g/l sucrose), 2 (MS + 30 g/l sucrose + 1 mg/l NAA) and 5 (½MS + 30 g/l sucrose + 1 mg/l NAA) media (Table 4), rooting was 95%. The lowest rooting created in media 6 including ½MS + 30 g/l sucrose + 0.5 mg/l NAA was 45%. The seedlings of shoot explant had the highest rooting. The results of regeneration indicated that direct regeneration was obtained from nodal explants (Figure 4). In 12 used media including 1, 5, 7, 8, 10, 11, 12, 13, 15, 17, 18, 19, the direct regeneration was 95%. The indirect regeneration was obtained from shoot, root and leaf explants (Figure 4).

Adaptation

The rooted plants were transferred to pots containing perlite for adaptation. Pots were covered for 3 weeks with plastic to save of high humidity. Temperature for adaptation was 22 ± 2º. After 3 weeks the plants in pots were adapted (Figure 1G).

Intra shoot variability of the shoot forming capacity

The shoot- forming capacity difference was determined from six shoot positions. In Nantes variety when auxin (2 mg/l NAA) alone was added, the positions nearer the apex showed higher capacity than the lower part, but with both regulators including auxin and cytokinin (2 mg/l NAA and 1 mg/l BAP) central or lower zones of the shoot had higher shoot- forming capacity (Table 6). Therefore, the shoot- forming capacity in the Nantes depended on the shoot position.

Table 6. Number of shoot-forming of shoot explants

		Used media for shooting				
Number of shoot	Posision of shoot explant	1	2	6	9	15
20	Nearer the apex	14	5	18	11	2
20	Central or lower zones	18	4	12	8	4

Shoot differentiation from leaf tissue

The results indicated the position of leaf efficient on shoot-forming capacity. Highest shoot- forming capacity was obtained from the two nearest explants to apex of 1 mg/l NAA of 2 mg/l (Table 7).

Table 7. Number of shoot-forming of leaf explants

		Used media for shooting				
Number of shoots	Posision of shoot explant	1	2	7	8	9
20	Nearer the apex	5	15	13	5	1
20	Furthest the apex	3	10	9	2	1

Genetic fidelity of regenerated plants of carrot

To evaluate genetic stability, 18 ISSR primers was used in this experiment. All ISSR profiles were monomorphic among regeneration plants and mother plant except for MS + 30 g/l sucrose + 0.5 mg/l 2,4-D treatment. This treatment was polymorphic with the mother plant with ISSR 10 marker (Figure 5).

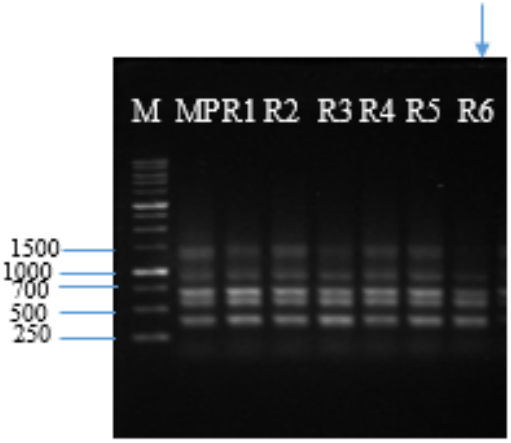

Figure 5. Genetic fidelity assessment of carrot with ISSR marker; profiles were obtained with ISSR 10 primer in carrot plant. The amplification products were monomorphic across all the regenerates obtained by organogenesis compared with the mother, except for R6 that showed genetic instability. M denotes marker, MP mother plant, and R1– 6 regenerated plantlets.

Discussion

Carrot plant is a valuable plant in nutritional and pharmaceutical fields. Because of the benefits of the carrot plant, it is used as a model plant for producing vaccines, recombinant proteins, molecular farming and genetic engineering. For this purpose, the first step is optimizing the carrot plant tissue culture (callus induction, embryogenesis, shooting). In this research, the increase in the regeneration of carrot plant was observed compared with previous studies [10, 13, 23, 24, 25].

The results of this study showed plantlets developed after two weeks in ½ MS medium. In Sundararajan *et al.*'s study, plantlet developed normal roots and shoots after three weeks in MS medium [25]. Tavares *et al.,* cultured seed in ½ MS and after 3- 4 weeks plantlet developed [12]. Sundararajan *et al.,* used *D. carota* L., cv. Kurado [25], Tavares *et al.,* used *D. carota* subsp. *Halophilus* [12] and Kamada *et al.,* used *Daucus carota* L. cv. US-Harumakigosun [28]. However, used variety in this research was Nantes.

The percentage of callus induction in this research was 95%. But in the research by Rabiei *et al.,* percentage of callugenesis was reported by 90% [10] and it was 75% in the study by Pant *et al*. [13, 25]. In this study, it was found that lower level of 2,4-D increased callus induction and use of NAA and BAP together increased percentage of callus induction. This result was similar to that of previous research [13, 25]. We used 2 mg/l NAA for callus induction, but Pant *et al*. and Rabiei *et al.* obtained high callus induction by 1 mg/l NAA and 0.2 mg/l 2,4-D, respectively. The difference in the result of previous studies and this study lies in the use of different plant growth

regulatory and different concentration of plant growth regulatory [10, 13].

The highest percentage of embryogenesis in the studies of Pant *et al*., Chen *et al.* and Yeu Yau *et al,.* were 72%, whereas the rate of embryogenesis in this study was 90%, which was more than that of the previous research [13, 27]. Further, there was a difference in the genotype and varieties (10). Also, in past research, embryogenesis was obtained by using 0.2 mg/l 2,4-D, while this experiment used 2 mg/l NAA [10]. Moreover, in this study, the medium used for embryogenesis was MS medium, whereas Rabiei *et al.,* used MS modification (MSm) medium for regeneration [10]. Kiszczak *et al.,* used 2,4-D and NAA at 0.1 $g{\cdot}L^{-1}$ for regeneration in B5 medium [14]. Another research used the NLN-13 medium supplemented with 0.2 mg/L 2,4D and 0.2mg/L kinetin for embryogenesis and 0.1 mg/L of kinetin for regeneration with microspore culture [15]. In a recent report, anther was applied for regeneration [16] and root [17]. It was found that decreasing level of 2,4-D increased embryogenesis and increased level of 2,4-D increased callus growth. Also, the use of low levels of BAP and 2,4-D increased the embryogenesis. The results of this study were similar to those of Rabiei *et al.* [10]. Similarly, Sundararajan *et al*., reported that 1 mg/l 2,4-D had the highest effect on embryogenesis and 2 mg/l 2,4-D had the lowest effect on embryogenesis [25].

The maximum percentage of shooting in past research was 60% [10, 25], while in this research, the percentage of shooting was 90%. In this inquiry, the best result for shooting was obtained from 2 mg/l NAA, while Rabiei *et al.,* used 0.1 mg/l Kinetin [10, 28]. The results showed that low concentrations of kinetin and 2,4-D were needed for the shooting [10, 13].

Unlike the results reported by Pant *et al*., Chen *et al.* and Yeu Yau *et al.,* on the percentage of rooting by 46 to 60% [13, 27, 28], this investigation displayed the percentage of rooting by 95%. For rooting, we used MS medium, whereas Yuan-Yeu Yau used B5 medium [28]. In previous research, 0.5 mg/l kinetin was added to medium for rooting [10], but in this research we used 1 mg/l NAA. These can be the reason for the difference in the rooting percentage. Most of the work on tissue culture established that auxin like IAA, IBA, NAA induced root formation while cytokinin like BAP, kinetin induced shoots and bud formation [10]. Similarly, in the present study, the use of BAP alone and combined with NAA showed initiation of multiple shoots from the nodal explant of *D. carota. In vitro* propagation by using different explant and different propagation methods report in carrot with Kim *et al.,* and Joshi *et al.,* was 75% [23, 24].

Studies stated above were about indirect regeneration. Pant *et al.* in 2007 used 1 mg/l NAA for direct regeneration of nodal explants in carrot [13]. Our result on the direct regeneration is in agreement with that of Pant *et al.* Also, multiple shoot was obtained from 2 mg/l BAP and 1 mg/l NAA using the nodal explants in carrot [13]. Grzebelus *et al.,* (2012) used hypocotyl and leaf explants for direct regeneration [31]. Also, it was shown that high efficiency of direct somatic embryogenesis was obtained in carrot protoplast culture with thin alginate layer system, simplified by Kao and Michayluk medium [32].

In previous research, the concentration of cytokinin efficient of auxin was reported, but in this research, using auxin (1 mg/l NAA) alone was efficient for shooting. In this research it was indicated that using different cytokinins and auxins, shoot-forming capacity of shoot explants was obtained of 2 mg/l NAA [29]. The highest shoot differentiation from leaf explants was obtained from 1 mg/l or 2 mg/l NAA [30]. Ohki showed the best shoot formation was obtained from hypocotyle and leaf explant in tomato. Shoot formation by hypocotyle explants was observed with NAA and BAP [29].

The results of this study indicated that genetic stability with ISSR primers showed high genetic fidelity in mother plant and regeneration plants in carrot in somatic embryogenesis (Figure 5). Bradeen *et al.,* used AFLP and ISSR for the analysis of molecular diversity of cultivated carrot (*Daucus carota* L.). The previous study

reported that variability was dependent on the genome, organs, position of organ in plantlet, maturity of organ on plantlet and kind and concentration of plant growth regulatory [20,21,29]. Wild *Daucus,* a variety of molecular marker was extensive between cultivated carrot and wild *Daucus* and relatively non-structured in nature because stress in nature is high. For this reason, genetic stability in wild carrot is low [33]. A comparison made between carrot genotypes study by ISSR and RAPD markers indicated high diversity in old varieties because natural selection in nature is high [34].

In this study, the cause of diversity in one regeneration plant was somaclonal variation in somatic embryogenesis in tissue culture. The reason for somaclonal variation is in vitro culture systems. Cell division and cell wall formation in somatic embryogenesis was higher than that of direct organogenesis. For this reason, somaclonal variation in somatic embryogenesis was higher than that of direct organogenesis [35,36].

The previous study indicated that variability was dependent on the genome, organs, position of organ in plantlet, maturity of organ on plantlet and kind and concentration of plant growth regulatory [29].

Conclusion

In this research, the best treatment for direct shoot regeneration was obtained from MS medium + 1 mg/l BAP + 2 mg/l NAA, and indirect regeneration (callus induction, embryogenesis and shooting) was obtained from MS medium containing 2 mg/l NAA. The highest rooting was recorded on the MS and ½MS medium, both containing 1 mg/l NAA. Intra shoot variability of the shoot forming capacity indicated in the Nantes depended on the shoot position. Shoot differentiation from leaf tissue indicated that the highest shoot- forming capacity was obtained from two nearest explants to apex of 1 mg/l NAA of 2 mg/l. The result of genetic stability indicated that it was very high in mother plant and regeneration plants. The novelty of this research lies in gaining higher callus induction, embryo-building and shooting compared with past research.

1. Implications for policy makers

Carrot is a plant model for researching molecular farming and vaccine production. For this reason, optimization of carrot is necessary and the first step for this goal. In the current research, the highest of callugenesis, embryogenesis and shooting were obtained.

2. Implications for public

For genetic manipulation in plant, the first step is optimization of tissue culture. Carrot as a plant is the model used for this purpose. The goal in this research was to obtain the highest callugenesis, embryogenesis and shooting.

Declarations

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Financial interests

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Conflicts of interests/ Competing interests

The authors have no relevant financial or non-financial interests to disclose. The authors have no conflicts of interest to declare that are relevant to the content of this article. All authors certify that they have no affiliations with or involvement in any organization or entity with any financial interest or non-financial interest in the subject matter or materials discussed in this manuscript. The authors have no financial or proprietary interests in any material discussed in this article.

Availability of data and material

All data is available.

Code availability

All code is available.

Ethics approval

Ethics approval have been observed.

Consent to participate

All writers have consent to participate.

Consent for publication

All writers have consent to publication.

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